PNAs grafted with (α/γ, *R*/*S*)-aminomethylene pendants: Regio and stereo specific effects on DNA binding and improved cell uptake†

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Received 20th September 2010, Accepted 27th October 2010
DOI: 10.1039/c0cc03988h

PNAs grafted with cationic aminomethylene (*am*) pendants on the backbone at the glycyl (α) or ethylenediamine (γ) segments show regio (α/γ) and stereochemistry (*R*/*S*) dependent binding with complementary DNA. These are efficiently taken up by cells, with γ(*S*-*am*) *aeg*-PNA being the best in all properties.

Peptide Nucleic Acid (PNA)[1] (Fig. 1a) has emerged as a promising class of oligonucleotide mimics with potential therapeutic and diagnostic applications. In PNA, the neutral and structurally homomorphous pseudo-peptide backbone (aminoethylglycine, *aeg*) is linked to the bases A, C, T and G. PNA hybridizes to complementary DNA and RNA with high affinity and selectivity is mediated by Watson–Crick base pairing. The unnatural polyamide linkage also makes it resistant to enzymatic degradation.[1] The simplicity of its structure with distinguishable properties, ease of synthesis and low toxicity has made *aeg*-PNA attractive as a putative genetic regulatory agent.[2] But its application as an effective antisense drug is limited by its low aqueous solubility, ambiguity in parallel or anti parallel[3] orientation of binding the target DNA or RNA and its uptake by mammalian cells. This has lead to a wide variety of chemical modifications.[3,4]

Incorporation of a positive charge in PNA variants carrying chiral D/L-lysine units in place of glycine, has shown promising properties in terms of improved solubility and higher binding of single-stranded DNA.[5] X-ray structure analysis of D-lysine based PNA indicated the backbone oxygen atom of the amide carbonyl to be in proximity of γ-carbon of the side chain (3.17 ± 0.1 Å).[6] Based on this, Topham *et al.*[7] predicted from theoretical calculations that a PNA backbone composed of 3-amino-D-alanine should promote preferential formation of a P-form helix through an intramolecular hydrogen bond between the side chain amino function at the α-C and the amide carbonyl of the backbone. Recent reports have indicated that L-lysine[8] and L-serine[9] derived γ-substituted PNA units within unmodified PNA oligomers act as helical directors due to conformational pre-organization. It has also been reported that cationic guanidinium groups grafted on PNAs (GPNA)[10] and the phosphate backbone of oligonucleotides[11] enhance the cell uptake of the substrates.

In this context we proposed to synthesise PNAs with chiral and cationic backbones consisting of aminomethylene (*am*) substituents either at the α-C of glycine (Fig. 1b and 1c) or on the γ-C of the ethylenediamine (Fig. 1d) fragments. The shorter side chain with one methylene reduces the non-specific electrostatic interactions in the derived PNA–DNA duplexes and yet still being cationic may also enhance cell uptake.

We herein report the synthesis of α-(*S*/*R*-*am*) *aeg*-PNA-T monomers (**1**,**2**) and a γ-(*S*-*am*) *aeg*-PNA-T monomer **3** (Scheme 1) starting from D/L-aspargine and their incorporation into PNA oligomers **P2–P4** (Table 1). It is found that γ-(*S*-*am*) *aeg*-PNA **P4** binds to complementary DNA very strongly and taken up in HeLa cells better than the α-(*R*/*S*-*am*) analogues.

PNA oligomers (**P2–P4**) incorporating the aminomethylene side chains at the α or γ positions as pendant groups on the backbone were made from the appropriate monomer, **1–3**, which in turn were synthesised from L or D-asparagine (**4a**/**b**) according to Scheme 1. The side chain α-aminomethylene group was generated from reduction of the amide in L or D-aspargine using iodobenzenediacetate[12] to give **5**. This was transformed to the fully protected **6**, which is the common intermediate to reach both the α and γ monomers **1–3** through two different synthetic paths. Route A gave the α-(*S*/*R*)-*am* monomers **1** and **2**, while route B lead to γ-(*S*)-*am* analogue **3** employing the reagent sequences shown in Scheme 1 (details in ESI†). Stereochemically, L-aspargine (**4a**) leads to α-(*S*) **1** and γ-(*S*) **3** monomers, while D-aspargine (**4b**) gave the α-(*R*) monomer (**2**).

The monomers **1–3** were incorporated at the desired sites (denoted by lowercase letters in Table 1) within the *aeg*-PNA sequence **P1** *via* solid phase synthesis using MBHA resin (see ESI†) to obtain *am*-PNA decamers **P2–P4**, each having two modified sites. The corresponding fluorescent oligomers cf**P1**–cf**P4** needed for cell uptake assays were synthesised by coupling 5- and 6-carboxyfluorescein at the N-terminus of the PNA oligomers before cleavage from the resin. All PNA oligomers were purified by HPLC, characterized by MALDI-TOF and used further for DNA hybridization and cell uptake studies.

Inspite of the presence of chiral centres, α-(*R*/*S*)-*am* single stranded PNAs **P2** and **P3** did not show appreciable CD signals. However, the γ-(*S*-*am*) *aeg* PNA oligomer **P4** exhibited a prominent negative CD signal at 273 nm suggesting a considerable helical pre-organization induced *via* base stacking (see ESI†). The complexing ability of *aeg*-PNA **P1** and the chiral, cationic (α/γ-*am*)-PNA oligomers **P2–P4** with the 10-mer complementary DNA **D1** (*antiparallel*), **D2** (*parallel*) and DNA **D3** (single C : C mismatch) were assessed by temperature dependent UV-absorbance experiments. The stoichiometry of the PNA : DNA complexes was found to be 1 : 1 by CD Jobs

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† Electronic supplementary information (ESI) available: Experimental procedures for synthesis and cell uptake experiments, MTT assay, ^{1}H and ^{13}C NMR of synthetic intermediates, HPLC, MALDI-TOF spectra of PNA oligomers, CD Jobs plot, UV-T_m data, cell uptake results. See DOI: 10.1039/c0cc03988h

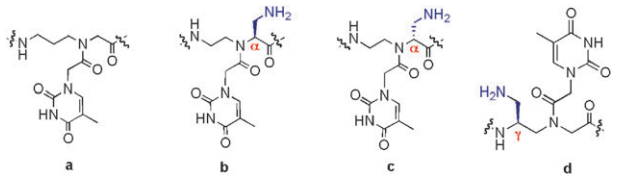
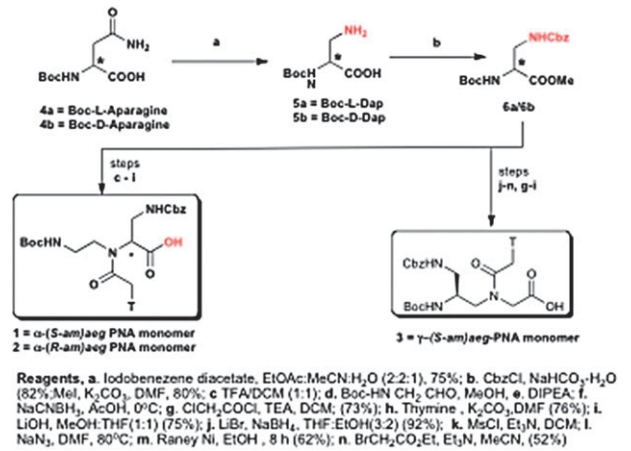


Fig. 1 Structures of PNAs (**a**) *aeg*-PNA (**b**) α(*S-am*) *aeg*-PNA (**c**) α(*R-am*) *aeg*-PNA and (**d**) γ(*S-am*) *aeg*-PNA.



Reagents, a. Iodobenezene diacetate, EtOAc:MeCN:H_2O (2:2:1), 75%; b. CbzCl, $NaHCO_3$-H_2O (82%;MeI, K_2CO_3, DMF, 80%; c TFA/DCM (1:1); d. Boc-HN CH_2 CHO, MeOH, e. DIPEA; f. $NaCNBH_3$, AcOH, 0°C; g. $ClCH_2COCl$, TEA, DCM; (73%); h. Thymine , K_2CO_3,DMF (76%); i. LiOH, MeOH:THF(1:1) (75%); j. LiBr, $NaBH_4$, THF:EtOH(3:2) (92%); k. MsCl, Et_3N, DCM; l. NaN_3, DMF, 80°C; m. Raney Ni, EtOH , 8 h (62%); n. $BrCH_2CO_2Et$, Et_3N, MeCN, (52%)

Scheme 1 Synthesis of α-(*S*/*R-am*) **1**,**2** and γ-(*S-am*) **3** PNA monomers.

Table 1 PNA sequences with *am* modifications

PNA		Sequence
aeg	**P1**	H-T T A C C T C A G T-lys NH_2
α-(*S-am*)	**P2**	H-T $\underline{t}^{\alpha(S)}$ A C C $\underline{t}^{\alpha(S)}$ C A G T-lys NH_2
α-(*R-am*)	**P3**	H-T $\underline{t}^{\alpha(R)}$ A C C $\underline{t}^{\alpha(R)}$ C A G T-lys NH_2
γ-(*S-am*)	**P4**	H-T $\underline{t}^{\gamma(S)}$A C C $\underline{t}^{\gamma(S)}$C A G T-lys NH_2
aeg PNA	cf**P1**	H-***CF***-T T A C C T C A G T-lys NH_2
α-(*S-am*)	cf**P2**	H-***CF***-T$t^{\alpha(S)}$ A C C$t^{\alpha(S)}$ C A G T-lys NH_2
α-(*R-am*)	cf**P3**	H-***CF***-T $t^{\alpha(R)}$A C C $t^{\alpha(R)}$ C A G T-lys NH_2
γ-(*S-am*)	cf**P4**	H-***CF***-T $\underline{t}^{\gamma(S)}$ A C C $\underline{t}^{\gamma(S)}$ C A G T-lys NH_2

$\underline{t}^{\alpha(S)}$ = α-(*S-am*) *aeg*-PNA, $\underline{t}^{\alpha(R)}$ = α-(*R-am*) *aeg*-PNA, $\underline{t}^{\gamma(S)}$ = γ-(*S-am*) *aeg*-PNA, ***CF*** = carboxyfluorescein.

plot (see ESI †) as expected for a duplex. The melting temperature T_m of PNA–DNA hybrids obtained from the UV data are shown in Table 2. The comparative differential T_m (ΔT_m) computed for modified **P2–P4** oligomers in relation to unmodified **P1** and for analogous antiparallel, parallel and mismatch PNA : DNA duplexes are shown in Fig. 2.

Table 2 UV-T_m (°C) studies of *aeg* PNA and *am*-PNA : DNA duplexes[a]

PNA	No	DNA **D1**(*ap*)	DNA **D2**(*p*)	DNA **D3**(*ap*)
aeg PNA	**P1**	49.2	48.5	42.2
α-(*S-am*)	**P2**	54.4	50.6	42.5
α-(*R-am*)	**P3**	57.7	48.6	41.0
γ-(*S-am*)	**P4**	62.3	49.7	44.0

[a] *ap*, *antiparallel* DNA **D1** = 5′ACTGAGGTAA3′; *p*, *parallel* DNA **D2** = 5′AATGGAGTCA3′; C–C base pair mismatch DNA **D3** = 5′ACTGC̲GGTAA3′. *Parallel*: PNA (N → C) : DNA(5′ → 3′); *antiparallel* PNA (N → C) : DNA(3′ → 5′).

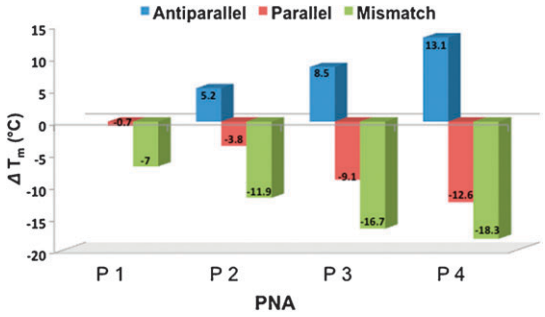


Fig. 2 Comparative ΔT_m values for hybrids of *aeg*-PNA **P1**, α-(*S-am*)-PNA **P2**, α-(*R-am*)-PNA **P3** and γ-(*S-am*)-PNA **P4** with DNAs **D1–D3**. (+) and (−) signs of ΔT_m correspond to stabilisation and destabilisation of duplexes, respectively.

The T_m data in Table 2 indicates that the cationic *am*-PNAs (**P2–P4**) form more stable duplexes than the unmodified *aeg*-PNA **P1** with the complementary DNAs **D1** and **D2**. Among the α-*am*-PNA : DNA **D1** antiparallel duplexes, the (*R-am*)-PNA **P3** had a T_m (4.3 °C-modification) higher than that of (*S-am*)-PNA **P2** (2.6 °C-modification). The γ-(*S-am*) *aeg*-PNA **P4** stabilised the antiparallel duplex most with DNA **D1** (6.5 °C-modification). It may be pointed out that the T_m enhancements seen with the present *am*-PNA modifications are better than ~2.0 °C-modification seen with γ-guanidine-PNA.[6] The results indicate that the shorter cationic side chains enhance binding significantly probably due to steric factors.

The orientational selectivity in binding by the chiral PNAs, was examined using DNA **D2** that can form *parallel* duplexes. The unmodified PNA **P1** does not show much difference in T_m of antiparallel and parallel duplexes. The α-(*R-am*)-PNA **P3** destabilised the parallel duplex (ΔT_m = −9.1 °C) more than the α-(*S-am*)-PNA **P2** (ΔT_m = −3.8 °C) compared to the corresponding antiparallel duplexes. However, γ-(*S-am*)-PNA **P4** was even better at stabilising the antiparallel duplex (ΔT_m = −12.6 °C) than the parallel duplex. Thus γ-(*S-am*) *aeg*-PNA **P4** not only shows a higher duplex stability with DNA, but also possess a better orientational selectivity than the α-(*R*/*S-am*) modifications.

The sequence specificity of binding of cationic *am*-PNAs was tested with DNA **D3** having one base C : C mismatching the middle of the antiparallel PNA : DNA duplex (Fig. 2, green bars). While the T_m of the single mismatch duplex from unmodified *aeg*-PNA **P1** was lower by 7 °C, for *am*-PNAs, the destabilization increased in the order **P2**, **P3** and **P4**. This data indicated that the higher binding affinity of the cationic PNAs *am*-PNAs **P2–P4** with anionic DNA, was achieved without sacrificing the sequence specificity. Both the site-specificity (α/γ) and the stereochemistry (*R*/*S*) of aminomethylene side chains seem to be important determinants in defining the stability of derived PNA : DNA duplexes.

The uptake of fluorophore labelled cationic PNAs cf**P1**–cf**P4** in HeLa cancer cells was measured with cells cultured on adherent monolayers in D-MEM media, incubated separately with each cf-PNA (1 μM) for 24 h. After washing with PBS, cells were imaged after fixing (see ESI†). The control cells not treated with PNA were stained with DAPI, a known dye for nuclear staining (Fig. 3). It is seen from the images that

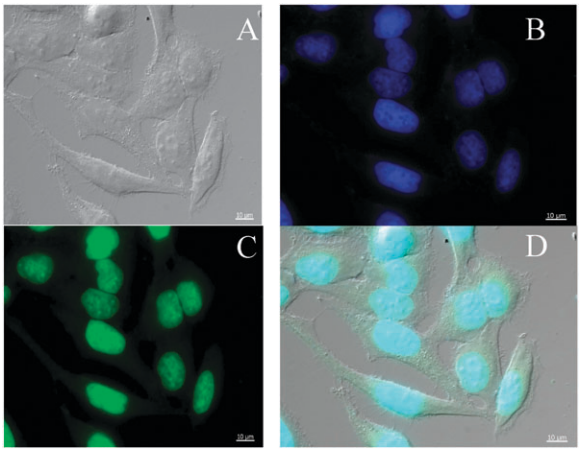


Fig. 3 Nuclear localisation of PNAs in cells (A) Differential Interference Contrast (DIC) image of cells (B) Cells stained with DAPI (C) Cells treated with cf**P4** and (D) overlapping of B and C.

cf-PNA **P4** is localised in the nucleus similar to DAPI (Fig. 3C) and confirmed from the super imposed images (Fig. 3D). cfPNAs **P1–P3** also showed similar behaviour even at the lowest of concentrations studied (0.25 μM). The results indicated that although all PNAs could enter the nucleus, the true effects of the cationic *am*-PNAs could not be distinguished.

The live cells were analysed by flow cytometry (FACS) after incubation with cf-PNAs **P1–P4** at 4 °C and 37 °C (Fig. 4). As seen from the fluorescence intensities, which are proportional to relative uptake efficiency, at 37 °C *aeg*-PNA cf**P1** and α-(*S-am*)-PNA cf**P2** showed only modest uptake in equal amounts followed by a slightly higher uptake of α-(*R-am*)-PNA cf**P3** and a maximum uptake of γ(*S-am*)-PNA cf**P4**. In comparison, at a lower temperature of 4 °C, the fluorescence intensities were 10 fold higher for all PNAs compared to those at 37 °C and the cell count was lower. At a higher temperature, the number of cells incorporating the fluorescent PNA was higher by 2 fold, but the intensity of fluorescence was lower, perhaps due to quenching effects. The cellular toxicity of the *am*-PNAs as measured by an MTT assay at different concentrations (see ESI†), showed the percentage viability to be more than 80%. These results indicate overall that not only do the pendant aminomethylene cationic charges on the PNA backbone improve the cell uptake but also their regio and

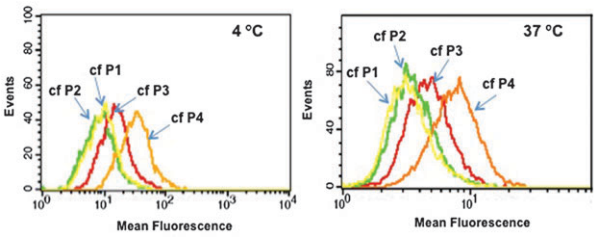


Fig. 4 FACS analysis of HeLa cells at 37 °C and 4 °C following incubation with cfPNA oligomers (cf**P1**–cf**P4**) (1 μM) for 24 h.

stereospecificity influences the efficiency of the cell uptake. The temperature experiments suggest this process to be energy dependent.

In conclusion, we have presented here the design and synthesis of regio (α/γ) and stereo specifically (*R*/*S*) modified PNAs carrying shorter cationic aminomethylene side chains on the backbone. Biophysical studies of *am*-PNAs show that the γ-(*S-am*)-PNA **P4** has the best sequence selectivity and the best DNA duplex stabilising properties. The cationic *am*-PNAs are taken up in cells better than the unmodified PNA **P1** with the γ-(*S-am*)-PNA **P4** being most efficient. It is also significant that cell uptake depends not only the site of modification, but also on its stereochemistry. The combination of highly sequence specific DNA binding and cell uptake properties along with their non-toxicity are very desirable attributes of cationic *am*-PNAs for their future potential applications. The amino function on the backbone will also be useful for conjugation with other functional and reporter molecules.[13] Future work in terms of increasing the cationic content, RNA complexing and cell-type specific uptake abilities are in progress.

RM thanks UGC (New Delhi) for award of a research fellowship. KNG is a recipient of JC Bose Fellowship from DST (New Delhi). We thank Prof Sanjeev Galande and Dr Mayurika Lahiri (IISER Pune) for their helpful discussions and assistance in the biological experiments.

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